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# Characterization of *Pectobacterium* strains causing soft rot and blackleg of potato in Finland

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ACADEMIC DISSERTATION

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# ABSTRACT

The aim of this dissertation was to characterize *Pectobacterium* strains isolated in Finland. *Pectobacterium* species cause soft rot and blackleg on wide range of plants in cultivated areas worldwide. Potato is an important food crop and source of food cultivated all over the world. *Pectobacterium* species interfere with potato production at all stages of cultivation. *Pectobacterium* species belong to the *Pectobacteriaceae* family with the soft rot bacteria *Dickeya* genus. In this study, two *Pectobacterium* model strains, SCC3193 and SCC1, isolated in Finland during 80s and initially defined as belonging to *P. carotovorum* species were further examined. Biochemical tests of the strains were conducted to understand their characteristics of the bacterial strains and their differences to closely related bacterial strains. The strain SCC3193, originally determined as belonging to *P. carotovorum*, was redefined as *P. wasabiae* in the present study. However, it did not fully share the same biochemical profile with the *P. wasabiae* type strain and based on genome comparisons it was later placed into a novel species *P. parmentieri*. Furthermore, phylogenetic position of the *Pectobacterium* strain SCC1 was determined. Also, the strain SCC1 was originally defined as *P. carotovorum*, but it was observed in the phylogenetic analysis that it clustered apart from *P. carotovorum* type strain, and thus its taxonomical status could not be confirmed at the time of the analysis. It was later included into a novel species called *Pectobacterium versatile*. In addition, *Pectobacterium* strains isolated from diseased potato stems in 2004, and initially classified as *P. carotovorum*, were characterized in this study. According to biochemical analyzes, these bacteria isolated from potato stems resembled *P. carotovorum* but had a low virulence on potato tuber and citrate-negative phenotype. Two genomes of these atypical Finnish stem isolates were produced to study their genome content and phylogenetic position in *Pectobacterium* genus. Average nucleotide identity (ANI) analysis showed that these isolates were similar to *Pectobacterium polaris*, a highly virulent new species recently identified in Norway. However, the Finnish isolates were most similar to atypical *P. polaris* isolates in ANI analysis and biochemical tests. Genome comparisons showed that the all the atypical isolates harbored similar genomic islands not present in *P. polaris* type strain. Alltogether, taxonomic and genomic studies placed the atypical *P. polaris* strains into a new subspecies, here called *P. polaris* subsp. *parvum*. One of the *P. polaris* subsp. *parvum* strains had been isolated in the Netherlands already in 1970s, but originally misidentified as *P. carotovotum*, which suggests that similar isolates were present in Europe also before.

This study provides novel information about the taxonomy and ecology of *Pectobacterium* species existing in Finland. Taxonomic status of *P. carotovorum* isolates redefined in this and other studies show that *Pectobacterium* strains previously included into *P. carotovorum* species could be divided into several novel species with genome-based methods. The precise identification of bacterial species poses challenges for plant protection. The information from the study can be used for potato production and plant protection in the future.

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscript that are reprinted and can be found at the end of this book with the permission of copyright holders:

I Nykyri, J., Niemi, O., Koskinen, P., Nokso-Koivisto, J., Pasanen, M., Broberg, M., Plyusnin, I., Törönen, P., Holm, L., Pirhonen, M. and Palva, E., T. 2012. Revised phylogeny and novel horizontally acquired virulence determinants of the model soft rot phytopathogen *Pectobacterium wasabiae* SCC3193. PLoS Pathogens 8(11):e1003013.

II Niemi, O., Laine, P., Koskinen, P., Pasanen, M., Pennanen, V., Harjunpää, H., Nykyri, J., Holm, L., Paulin, L., Auvinen, P., Palva, E., T. and Pirhonen, M. 2017. Genome sequence of the model plant pathogen *Pectobacterium carotovorum* SCC1. Standards in Genomic Sciences 12:87.

III Pasanen, M., Laurila, J., Brader, G., Palva, E., T., Ahola, V., van der Wolf, J., Hannukkala, A. and Pirhonen, M. 2013. Characterisation of *Pectobacterium wasabiae* and *P. carotovorum* subsp. *carotovorum* isolates from diseased potato plants in Finland. Annals of Applied Biology 163:403–419.

IV Pasanen, M., Schott, T., Degefu, Y., Koskinen, P., Ahola, V., Cleenwerck, I., Vandamme, P., Woodward, M., Pritchard, L. and Pirhonen, M. Improved characterization of *Pectobacterium polaris* and proposal of *P. polaris* subsp. *parvum* nov. (manuscript)

The publications are referred in the following text with their Roman numerals.

# ABBREVIATIONS

| | |
|---|---|
| AHL | *N*-Acyl homoserine lactone |
| ANI | Average nucleotide identity |
| BLAST | Basic Local Alignment Search Tool |
| Cel | Cellulase |
| CVP | Cristal violet pectate |
| DDH | DNA-DNA hybridization |
| DNA | Deoxyribonucleic acid |
| ENA | European Nucleotide Archive |
| HR | Hypersensitive response |
| Hrp | Hypersensitive response and pathogenicity |
| ICNP | International Code of Nomenclature of Prokaryotes |
| IGS | Intergenic spacer |
| IJSEM | International Journal of Systematic and Evolutionary Microbiology |
| LPSN | The List of Prokaryotic Names with Standing in Nomenclature |
| MLSA | Multilocus sequence analysis |
| NCBI | National Center for Biotechnology Information |
| PCR | Polymerase chain reaction |
| Peh | Polygalactouronase |
| Pel | Pectate lyase |
| Pnl | Pectin lyase |
| Prt | Protease |
| SRP | Soft-rot pectobacteria |
| T1SS | Type I secretion system |
| T2SS | Type II secretion system |
| T3SS | Type III secretion system |
| T4SS | Type IV secretion system |
| T5SS | Type V secretion system |
| T6SS | Type VI secretion system |
| WGS | Whole genome sequence |

Key words: plant pathology, bacteriology, ecology, *Pectobacterium*, potato, soft-rot, blackleg

# 1. INTRODUCTION

Potato is the world's third most important food crop and also an important source of starch and it is cultivated in every continent except Antarctica. Potato yields vary across the world (Birch *et al*. 2012). In total, potato is produced 325 million tonnes per year in the world and the most of it, around 80%, is produced in Europe and Asia (FAO 2007). The biggest potato producing country is China with 72 million tonnes per year (FAO 2007). Almost half of the potatoes produced are consumed in Asia, although the consumption per person is 24 kg/year per person while it is around 88 kg/year per person in Europe (FAO 2007). In Finland, potato production has fallen in recent years. In 2005, 743 000 tonnes potatoes were produced, but ten years later the production was more than 100 000 tonnes less, about 611 900 tonnes (FAO 2015). The cultivated area has also decreased from 41000 hectares in 1990 to 21200 hectares in 2017 (FAO 2017), but on the other hand, new cultivars produce more yield.

Potato production is threatened by several different pathogens such as bacteria, fungi, viruses and mycoplasmas. Disease control is important for a higher and good quality crop. Soft rot *Pectobacteriaceae* (SRP), formerly soft rot *Enterobacteriaceae* (SRE), consist of the genera *Pectobacterium* and *Dickeya,* and they cause soft rot and blackleg on potato and rotting of vegetables, fruits and ornamental plants worldwide and extensive economic losses in the field and storage (Bhat *et al*. 2010, Adeolu *et al*. 2016). Collectively, soft rot bacteria are listed among the 10 economically most important plant pathogens globally (Mansfield *et al*. 2012). It has been estimated that economic losses caused by these bacteria in agriculture and horticulture varied US$50-100 million annually already early 80s (Pérombelon and Kelman 1980), and soft rot postharvest losses have been estimated to be 15-30% of the harvested crop (Agrios 2006). According to Essarts *et al*. (2016), *Pectobacterium* and *Dickeya* species are the main reason for the rejection and downgrading of potatoes in seed potato certification.

## 1.1. SOFT ROT AND BLACKLEG OF POTATO CAUSED BY *PECTOBACTERIUM* SPECIES

### 1.1.1. Symptoms on potato

*Pectobacterium* spp. cause blackleg and soft rot on potato stems and tubers (Pérombelon and Kelman 1980, Pérombelon 1992). The tuber soft rot infection usually originates from the lenticels, stolon end or wounds when the conditions are moist and wet (Czajkowski *et al*. 2011). Later the infection proceeds inside the potato tuber and the rotting potato becomes soft and slimy and sometimes potato-bacterial mass may burst out of the potato. In potato stems, symptoms are seen as rotting and black stem, and during dry weather as chlorotic and hanging leaves caused by the limited water and nutrient flow in the vascular system caused by the bacteria (Pérombelon and Kelman 1980). Diseased mother tuber may cause poor emergence and potato growth. Blackleg symptoms start from the mother tuber and spread later to the basal part of the stem and further into the stem of the potato plant, where the disease is visible as black and slimy lesions (Pérombelon and Kelman 1987).

### 1.1.2. Virulence proteins and their secretion

*Pectobacterium* species produce enzymes that break down the cell walls of the plants (Collmer and Keen, 1986, Davidsson *et al*. 2013). These secreted enzymes include pectinases (pectate lyases (Pel), pectin lyases (Pnl), polygalactouronases (Peh)), cellulases (Cel), and proteases (Prt) (Collmer and Keen 1986, Toth *et al*. 2003, Charkowski *et al*. 2012). *Pectobacterium* genomes also contain genes encoding other enzymes, such as pectin methylesterases, pectin acetylesterase, oligogalacturonide lyase, rhamnogalacuronate lyase, beta and alpha-glucosides (Nykyri *et al*. 2012, Li *et al.* 2019). The secreted enzymes are responsible for the degradation of middle lamella, which is composed of pectin and is responsible for keeping the plant cells together (Johnson *et al*. 2010). Inside the plant, bacteria can survive in the vascular tissue or the intercellular space, until the environmental conditions are optimal for the disease development (Toth *et al*. 2003).

Bacterial pathogens use several specialized secretion systems to transport various proteins out of the bacterial cell. These secreted proteins are important for bacterial pathogenesis (Salmond 1994, Xu and Liu 2014). Secretion systems I-VI have been identified in Gram-negative bacteria (Xu and Liu 2014). Genome comparison of 84 *Pectobacterium* genomes have revealed that all analysed genomes contain at least two Type I secretion systems (T1SS), one possibly involved in secretion of proteases, and the second involved in secretion of multi-repeat adhesin required for adhesion to host plant and virulence (Li *et al.* 2018, Pérez-Mendoza *et al.* 2011). Type II secretion system (T2SS) has an important

virulence function in Pectobacteria due to its involvement in secretion of cell wall degrading pectinases, cellulases and necrosis-inducing peptide (Nip) (Pirhonen *et al.* 1991, Toth *et al*. 2003, Laasik *et al.* 2014). In biotrophic bacteria such as *Pseudomonas syringae*, T3SS is involved in effector protein translocation into the plant cell to down-regulate plant defence to promote virulence (Alfano and Collmer 2004). If the effector protein is recognized by plant disease resistance protein, the recognition triggers hypersensitive response (HR) that is a defense mechanism visible as local cell death around the infection site, which prevents the spread of a the biotrophic pathogen (Morel and Dangl 1997, Alfano and Collmer 2004, Choi *et al*. 2013). *Pectobacterium* species are necrotrophs that multiply on dead tissue, and thus HR is beneficial for their virulence. Full virulence of *P. carotovorum, P. atrosepticum* and *P. versatile* on potato and tobacco leaves has been shown to depend on secretion of effector protein DspE by T3SS into the host tissues, where it induces necrosis and cell death that promotes virulence and growth of the pathogen (Rantakari *et al*. 2001, Holeva *et al*. 2004, Kim *et al*. 2011, Hogan *et al*. 2013). DspE is a core effector that has two effects on defense suppression and cell death. It is present in most plant pathogenic bacteria and is involved in suppressing plant defences, and this, together with its necrotic activity, has been linked to its involvement as a phosphatase activator (Degrave *et al*. 2015).

Compared to T1SS, T2SS and T3SS, much less is known about the other secretion systems and their role for the for the pathogenicity of *Pectobacterium* species. The type IV secretion system (T4SS) allows pathogens to transfer DNA and proteins across the bacterial cell wall (Christie *et al*. 2005). Mutation in a gene coding for T4SS has been shown to reduce virulence of *P. atrosepticum*, but the translocated proteins have not been identified (Bell *et al*. 2004). Type V secretion system (T5SS) is said to be the simplest secretion system in bacteria (Charkowski *et al*. 2012), because only 1-2 proteins are needed for the secretion machinery (Fan *et al*. 2016). However, T5SS genes have not been characterized in the *Pectobacterium* species. The type VI secretion system (T6SS) transports effector proteins into other bacterial cells (Coulthurst 2019), and according to Liu *et al*. (2008) and Nykyri *et al*. (2012), full virulence of *P. atrosepticum* and *P. parmentieri* in potato is dependent on it.

### 1.1.3. Quorum sensing in Pectobacteria

Quorum sensing (QS) system is a process in which bacteria sense their cell density and the concentration change of the signaling molecules called autoinducers (AIs) (Fuqua *et al*.

1994, Miller and Bassler 2001, Papenfort and Bassler 2016). The concentration of autoinducer molecules increases as the number of bacteria increases, and when a threshold level is reached, gene expression is altered (Miller and Bassler 2001, Papenfort and Bassler 2016). Most Gram-negative bacteria use N-acyl derivatives of homoserine lactone (acyl homoserine lactones, AHLs) and Gram-positive bacteria typically utilize oligopeptides and amino acids as their main autoinducer molecules (Whitehead *et al*. 2001, Papenfort and Bassler 2016). AHLs can diffuse freely through the cell membrane (Papenfort and Bassler 2016). The length of the acyl group of the AHL molecule varies from 4 to 18 carbons (Kumari *et al*. 2006), and the hydroxyl and carbonyl group may vary and there might be a carbon-carbon double bond, or the molecule may be fully saturated (Gera and Srivastava 2006). Differences between the bacterial isolates in the structures of the AHL molecules have been noted, but no systematic comparison of the AHLs produced by various *Pectobacterium* species have been conducted. Most *Pectobacterium* isolates produce one major AHL molecule and one minor AHL that is produced in lower concentration, and also trace amounts of additional AHL molecules have been identified from some isolates (Crépin *et al.* 2012). The major and minor AHL molecules produced by *Pectobacterium* isolates with verified taxonomic position are shown in Table 1.

QS regulates production of virulence proteins by plant pathogenic bacteria. Pectobacteria produce cell wall-degrading enzymes only when the population density reaches a certain threshold level (Barnard and Salmond 2007). Also, T3SS, necrosis-inducing protein (Nip), carbapenem antibiotic production and virulence regulators *expR*, *rsmA* and *virR* are regulated via QS system (Burr *et al*. 2006, Chatterjee *et al*. 1995, Cui *et al*. 2005, Mattinen *et al*. 2004, McGowan *et al*. 2005, Smadja *et al*. 2004).

Table 1. Major and minor autoinducer molecules produced by *Pectobacterium* strains having verified taxonomic position.

| Strain | Species | Major AHL molecule | Minor AHL molecule | Reference |
|---|---|---|---|---|
| CFBP 1526$^T$ | *P. atrosepticum* | 3-oxo-C8-HSL | 3-oxo-C6-HSL, C8-HSL | Crépin *et al.* 2012 |
| CFBP 6276 | *P. atrosepticum* | 3-oxo-C8-HSL | 3-oxo-C6-HSL, C8-HSL | Crépin *et al.* 2012 |
| ATCC39048* | *P. brasiliense* | 3-oxo-C6-HSL | | Bainton *et al.* 1992 |
| CFBP 2046$^T$ | *P. carotovorum* | 3-oxo-C6-HSL | 3-oxo-C8-HSL | Crépin *et al.* 2012 |
| SCC3193* | *P. parmentieri* | 3-oxo-C8-HSL | 3-oxo-C6-HSL | Brader *et al.* 2005 |
| s0421$^T$ | *P. polaris* subsp. | 3-oxo-C6-HSL | | Pasanen *et al.* 2013 |
| s0416 | *parvum* | 3-oxo-C6-HSL | | Pasanen *et al.* 2013 |
| SCC1* | *P. versatile* | 3-oxo-C6-HSL | 3-oxo-C8-HSL | Brader *et al.* 2005 |
| Ecc71* | *P. versatile* | 3-oxo-C6-HSL | | Cui *et al.* 2005 |

* The taxonomy of the isolates is according to Li *et al.* 2018 and Portier *et al.* 2019.

### 1.1.4. Spreading and survival of Pectobacteria

*Pectobacterium* spp. can live as endophytic, epiphytic or saprophytic in plants, on plant surfaces, in soil or in water (Pérombelon and Kelman 1980). There are several different ways in which *Pectobacterium* species can spread within and between plants. Contaminated seed potato is the most important source of bacteria. During the growth of the potato plant, bacteria can move from the mother tuber to the stems and along the stolons to the daughter tubers. Diseased tubers can spread the disease to healthy tubers during harvest and grading. Under storage conditions, the bacterium can remain dormant in tuber lenticels and wounds without any visible symptoms (Pérombelon 1992), but if the conditions are favorable for the growth of bacteria, the contaminated tubers can start rotting and spread the disease to large number of tubers very quickly (Pérombelon and Kelman 1980).

Rainwater acts as a source of contamination, because it transports bacteria in soil from rotten seed tubers to the surface of other tubers, where the bacteria can penetrate to the healthy tuber through lenticels and wounds. The bacterium can also be transported with

aerosols created by rain on the healthy plants (Graham and Harrison 1975, Graham *et al*. 1977). Karjalainen at al. (2000) stated that *Pectobacterium* spp. may stay viable on wet potato leaves and also multiply on them. Burgess *et al*. (1994) noticed that there was an increase in the amount of *Pectobacterium* spp. bacteria later in the growing season on potato leaves, from where the bacteria can spread to the tubers during harvest. Also, the contaminated soil can act as a source of bacteria to healthy seed tubers (Powelson and Apple 1984, Maher *et al*. 1986) and the pathogen can overwinter in plant residues left in the soil after harvest (Pérombelon 1973, De Boer *et al*. 1979). Pérombelon and Hyman (1987) investigated how soft-rot bacteria can spread from an infectious source in the river and they noticed that soft-rot bacteria could be isolated already after 2 hours and still after 15 days up to 8 km away from the site of the inoculated potatoes, which indicates that contaminated surface water can efficiently spread bacteria especially if it is used for irrigation.

Insects can spread the pathogen during the growing season (Molina *et al*. 1974, Kloepper *et al*. 1981, Pérombelon and Kelman 1980), as bacteria can remain viable in insects over long time (Karjalainen *et al*. 2000). Insects can serve as alternative hosts for plant pathogenic bacteria and can also act as vectors for pathogens. Fruit fly, *Drosophila melanogaster*, has been shown to carry and transmit *Pectobacterium* species (Molina *et al*. 1974, Kloepper *et al*. 1981), and blowfly *Chrysomya megacephala* and housefly *Musca domestica* have been shown to contain Pectobacteria as part of their microbiome (Junqueira *et al.* 2017). Recently, Rossmann *et al*. (2018) investigated insect species related to the spread of Pectobacteria and analyzed insect samples collected in Norway from symptomatic potato fields by quantitative PCR (qPCR) assay targeting several *Pectobacterium* species, and isolated virulent bacteria from the qPCR-positive insects. They noted that several insects can act as vectors due to their high bacterial content. The insects carrying the highest numbers of soft rot bacteria belonged to the genus *Delia* (*D. platura*, a seedcorn maggot, being the most common) and in total, 91 different insect species were found to carry soft rot bacteria (Rossmann *et al*. 2018). However, it is not known whether SRE are part of the natural microbiome of the insects or if they are true free living SRP.

### 1.1.5. Control strategies of *Pectobacterium* species

The production of healthy certified seed potatoes according to high standards is important for the commercial cultivation of potatoes. Fighting soft rot and blackleg is difficult because

there are no chemical control methods and no resistant cultivars (Karjalainen *et al*. 2000). To control the disease, it is important to know how the disease is dissiminated to prevent it from spreading further in the potato production chain and to identify latent bacterial infections (Karjalainen *et al*. 2000). Good cultivation techniques, the use of the healthy seed potato and avoiding of contamination have been used to prevent the spread of the disease. Cultivation techniques can reduce infections and the onset of symptoms (Lehtinen and Hannukkala 2004). It is important to use healthy seed material and cleaned tools and machinery in potato cultivation. In addition, cooling and drying of the potato tubers before storage and right storage conditions reduces the risk of the disease. Collection of diseased plants during the growing season reduces the spread of the disease to the healthy individuals.

It has been shown that calcium fertilization of potato field can slow down the decay of tubers caused by the *Pectobacterium* species (Karjalainen *et al*. 2000). According to the studies, the accumulation of calcium ions affects the structure of the cell wall and increase the ristance of the tubers to soft rot (Karjalainen *et al*. 2000, McGuire and Kelman 1986). Physical and chemical methods have been under study but with narrow success (Czajkowski *et al*. 2011). Also, several studies have been done to test biocontrol agents against soft-rot pathogens (Azaiez *et al*. 2017, Carstens *et al*. 2019, Cui *et al*. 2019, Muturi *et al*. 2019). Researchers have succeeded in developing phage-based biological control against bacteria and several phage biocontrol products have been introduced to the market in recent years (Agriphage by Omnilytics Inc., https://www.omnilytics.com/, ErwiPhage by Enviroinvest Zrt., www.erwiphage.com). APS Biocontrol Ltd., a Scottish company, has developed a bacteriophage-based wash solution (APS' Biolyse® mix) for potato tubers to be used during storage against Pectobacteria (https://www.apsbiocontrol.com/). Although various methods against Pectobacteria have been studied, the control of the disease after infection remains difficult.

Latent infections are difficult to detect from asymptomatic plants. Testing seed potatoes for the presence of soft rot bacteria is an important aspect in the production of high-quality seed potato. Early identification of the pathogen in seed potatoes used for cultivation prevents the spread of the disease. Seed potato lots are classified into different seed grades and highly contaminated potato lots are rejected. In Europe, seed potato certification has been used since the early 1900s (DeHaan 1994). The symptoms caused by the *Pectobacterium* and

*Dickeya* species are very similar and it is difficult to visually determine the causal agent of the disease in the field or in the storage. Seed potato certification laboratories have various tests for the identification of pathogens. In Finland, certified high-quality seed potatoes are produced mainly in Northern Ostrobothnia that is one of five High Grade Regions in EU (Luke 2018). Only regions or countries that do not have quarantine pests for potatoes or from which they have been eradicated may be designated as 'High Grade Region'. In addition to Finland, four member states or their regions (Portugal, Ireland, United Kingdom and Germany) have been granted the status of High Grade Region.

## 1.2. TAXONOMY OF *PECTOBACTERIUM* SPECIES

### 1.2.1. Identification of soft rot Erwinias

Bacterial soft rot was first described on carrots, and the isolated plant pathogenic bacterium was named as *Bacillus carotovorus* in 1901 (Jones 1901). One year later potato blackleg-causing bacterium was named *Bacillus atrosepticus* (van Hall 1902, Gardan *et al*. 2003). These two pathogens were classified as two distinct species based on the pathogenicity and host-plant origin. Winslow *et al*. (1917) were the first ones to suggest that peritrichous plant pathogenic bacteria should form a new genus called *Erwinia* after American plant pathologist Erwin F. Smith in the preliminary report of the Committee of the Society of American bacteriologist on characterization and classification of bacterial types published in 1917. Definition for the genus was plant pathogens, whitish and slimy growth, not usually producing indol and in certain carbohydrate media acid was usually formed (Winslow *et al*. 1917). Genus was placed in the family *Enterobacteriaceae*. Final report of the Committee was published in 1920 with *Erwinia amylovora* as the type strain of the genus *Erwinia* (Winslow *et al*. 1920).

Dye (1969) proposed division of soft rot Erwinias into varietes as *Erwinia carotovora* var. *carotovora*, *E. carotovora* var. *atroseptica* and *E. carotovora* var. *chrysanthemi.* After this, Brenner *et al*. (1973) added *E. chrysanthemi* and *E. rhapontici* into the genus *Erwinia*. During the 80s and 90s several new bacterial strains were added to the genus. At the early 1980s, Thomson *et al*. (1981) established a new subspecies of *Erwinia carotovora* containing bacterial strains isolated from diseased sugar beet plants in California. These bacteria were called *Erwinia carotovora* subsp. *betavasculorum.* Goto and Matsumoto (1987) isolated from

Japanese horseradish (*Eutrema wasabi* Maxim.) soft rot bacteria that differed in their biochemical properties from subspecies *carotovora, atroseptica* and *betavasculorum* and proposed the name *Erwinia carotovora* subsp. *wasabiae*. Similar strains have been also reported from potato plants in potato growing areas in the United States, New Zealand, Iran and South Africa (Kim *et al*. 2009, Pitman *et al*. 2010, Baghaee-Ravari *et al*. 2011, Moleleki *et al*. 2013). Alcorn *et al*. (1991) isolated soft-rot bacteria from diseased cactus plants and defined them as a new species of *Erwinia* genus, *Erwinia cacticida*. Soft rot of witloof chicory (*Cichorium intybus* L.) was first observed in France, and the isolated pathogen was first classified as atypical *Erwinia carotovora* subsp. *atroseptica* (Samson *et al.* 1980), but later a new subspecies, *Erwinia carotovora* subsp. *odorifera* was proposed (Gallois *et al*. 1992).

1.2.2. Identification of *Pectobacterium* genus

Waldee (1945) proposed a separate genus in the *Enterobacteriaceae* family named as *Pectobacterium*, which included strains causing soft rot on plants. These bacterial strains differed in their pathogenicity from the non-pectinolytic *Erwinia* species causing wilt and blight (Waldee 1945). *P. carotovorum* subsp. *atrosepticum* and *P. carotovorum* subsp. *carotovorum* and *P. cypripedii* were included into this genus. However, the name was not commonly adopted. The genus *Pectobacterium,* including species *P. carotovorum*, *P. chrysanthemi*, *P. cypripedii* and *P. rhapontici*, appeared for the first time on the Approved List of Bacterial names in 1980 (Skerman *et al*. 1980). Hauben *et al*. (1998) divided genus *Erwinia* into three phylogenetic groups - *Erwinia*, *Pectobacterium* and *Brenneria,* and soft rot pathogens were placed in the genus *Pectobacterium* as *P. carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *carotovorum, P. cacticidum* and *P. chrysanthemi*. These names were also validly published in 1999 when they appeared on Validation List no. 68 (Validation list 68, 1999). Later *Pectobacterium* genus was divided by Samson *et al*. (2005), who separated *P. chrysanthemi* (*E. chrysanthemi* Burkholder *et al*. 1953) from the genus *Pectobacterium* to the genus *Dickeya* gen. nov. In 2003, Gardan *et al*. (2003) refined the *Pectobacterium* taxonomy and proposed that *Pectobacterium* subspecies *atrosepticum*, *betavasculorum* and *wasabiae* should be elevated to a species level as *P. atrosepticum*, *P. betavasculorum* and *P. wasabiae*. *P. c*. subsp. *carotovorum* and *P. c*. subsp. *odoriferum* remained as subspecies. *P. brasiliense* was first isolated in Brazil from diseased potato plants (Duarte *et al*. 2004) but later observed to cause blackleg and tuber soft rot on potato worldwide (van der Merwe at al. 2010). After identification, it has been detected in Israel (Ma

*et al*. 2007), South Africa (van der Merwe *et al*. 2010), Canada (De Boer *et al*. 2012), New Zealand (Panda *et al*. 2012), Zimbabwe (Ngadze *et al*. 2012), the Netherlands (Leite *et al*. 2014), Italy (Cariddi and Bubici 2016), Mexico (Mejía-Sánchez *et al*. 2019), Russia (Voronina *et al*. 2019), China (Zhao *et al*. 2018) and in Finland (Yeshitila Degefu, personal communication). It can also infect carrot, *Capsicum annum* and *Ornithogalum* species (Nabhan *et al*. 2012).

Pectobacteria have also been identified as phytopathogens of fruit and ornamentals. Bacterial strains showing similar symptoms to those of *P*. *syringae* pv. *actinidiae* but having phenotypic differences were isolated from symptomatic kiwifruit plant *Actinidia chinensis* cv. Hort16A (Koh *et al*. 2012). Isolated bacteria had a distinct taxonomical position among the *P*. *carotovorum* subspecies and a novel subspecies *Pectobacterium carotovorum* subsp. *actinidiae* was proposed (Koh *et al*. 2012). Species infecting mainly monocotyledonous plants were proposed to belong to the novel species *Pectobacterium aroidearum* by Nabhan *et al* (2013). *P*. *aroidearum* has been shown to cause symptoms also on potato (Moretti *et al*. 2016). Moretti *et al*. showed that the strain LMG 2408 previously isolated from *Zantedeschia aethiopica* and identified as *P. c.* subsp. *carotovorum*, had been misclassified and was placed in *P. aroidearum* species.

1.2.3. Effect of genomic sequencing on *Pectobacterium* taxonomy

Over the past decade, as the sequencing costs has fallen, sequencing has become commonplace in taxonomic work. Khayi *et al*. (2016) re-evaluated the taxonomy of *P. wasabiae* strains isolated from potato by using whole genome sequences of the strains. These bacterial strains were shown to be present in Europe since 1980s, classified as *P. c.* subsp. *carotovorum,* but had been later reclassified to belong to *P*. *wasabiae* species (Pitman *et al*. 2010, Nykyri *et al*. 2012). In further studies by Khayi *et al*. (2016), isolates that had been identified from potato were separated from the Japanese horseradish isolates, and a new species *P*. *parmentieri* was proposed for the potato-infecting isolates. In 2017, Dees *et al*. (2017) reclassified *P. carotovorum* strains isolated in Norway, and proposed a novel highly virulent species, *Pectobacterium polaris* (Dees *et al*. 2017). These bacterial strains were isolated from infected potato tubers and originally defined as *P. c.* subsp. *carotovorum* (Dees *et al*. 2017). Sarfraz *et al*. (2018) used genomic and phenotypic comparisons to distinguish *Pectobacterium* strains isolated from potato plants in Pakistan.

Strains differed from their closest relatives *P. wasabiae* and *P. parmentieri* and other *Pectobacterium* species. The name *Pectobacterium punjabense* was proposed for the new species (Sarfraz *et al*. 2018).

In 2019, several new bacterial strains were described by various researchers. *Pectobacterium* strain M022, previously isolated from a waterfall source in Selangor district in Malaysia (Chan and Tan 2015), was reclassified and placed to a new clade *Pectobacterium fontis* (Oulghazi *et al*. 2019). *P. fontis* was able to cause rotting on potato tubers but it was less virulent than *P. parmentieri* and *P. brasiliense* (Oulghazi *et al*. 2019). Waleron *et al*. (2019a) published a new *Pectobacterium* species in 2019 isolated from the ground water from the vegetable field in North Poland and the name *Pectobacterium polonicum* was proposed, with *P*. *punjabense*, *P*. *parmentieri* and *P*. *wasabiae* being the closest relatives (Waleron *et al*. 2019b). Pédron *et al*. (2019) suggested a novel *Pectobacterium aquatium* species for the strains isolated from the waterways in South-East France. In addition, Waleron *et al*. 2019b isolated *Pectobacterium zantedeschiae* from calla lily bulbs and leaves. Plants were grown in Poland and Serbia, respectively. *P. zantedeschiae* does not resemble *Pectobacterium aroidearum* also having a preference towards monocotyledonous species (Waleron *et al*. 2019b). Furthermore, Shirshikov *et al*. (2018) proposed tentatively that a previously isolated Finnish strain SCC1 should be a type strain of a new species, Candidatus *Pectobacterium maceratum*, whereas Portier at al. (2019) renamed the species *Pectobacterium versatile*. According to Portier *et al*. (2019), the name *maceratum* is too universal to describe one single *Pectobacterium* species and name *Pectobacterium versatile* was proposed.

Taxonomical classification of the whole genera *Pectobacterium* has been revised various times in recent years. Zhang *et al*. (2016) re-evaluated the taxonomy of *Pectobacterium* species and suggested that the four *P. carotovorum* subspecies (*P. c.* subsp. *actinidiae*, *P. c.* subsp. *brasiliense, P. c.* subsp. *carotovorum* and *P. c.* subsp. *odoriferum*) should be elevetaed to species level. Portier at al. (2019) analyzed 110 *Pectobacterium* genomes utilizing genome comparisons and verified that the four subspecies of *P. carotovorum* subspecies represented individual species and renamed the remaining subspecies as *Pectobacterium odoriferum*, *Pectobacterium actinidiae* and *Pectobacterium brasiliense* (Portier *et al*. 2019). All *Pectobacterium* species, their host plants and the year when the

species was identified for the first time are listed in the Table 2. Due to the rapid change in *Pectobacterium* taxonomy, the species names in Table 2 may not reflect present taxonomy.

Table 2. *Pectobacterium* species, host plants from which the strains were isolated and year when the species were identified and published. Adapted from Charkowski (2018) with additional species added to the table. Taxonomic names of the *Pectobacterium* species are as reported by the authors and may not reflect present taxonomy.

| **Species** | **Host species** | **Year when first published** |
|---|---|---|
| *P. aquaticum* | environment/fresh water Pédron *et al*. 2019 | 2019 |
| *P. actinidiae* | *Actinidea chinensis* (kiwifruit) Koh *et al*. 2012<br>*Actinidia deliciosa* (kiwifruit) Wu *et al*. 2017 | 2012 |
| *P. aroidearum* | *Cucurbita pepo* (zucchini) Moraes *et al*. 2017<br>*Ornithogalum dubium* (sun star flower) Nabhan *et al*. 2013<br>*Persea americana* (avocado) Nabhan *et al*. 2013<br>*Saccharum spp.* (sugarcane) Nabhan *et al*. 2013<br>*Solanum tuberosum* (potato) Nabhan *et al*. 2013<br>*Zantedeschia aethiopica* (calla lily) Nabhan *et al*. 2013 | 2013 |
| *P. atrosepticum* | *Capsicum annuum* (pepper) Stommel *et al*. 1996<br>*Helianthus annuus* (sunflower) Baştaş *et al*. 2009<br>*Solanum melongena* (eggplant) Catara *et al*. 2001<br>*Solanum tuberosum* (potato) van Hall 1902 (Gardan *et al*. 2003)<br>*Zantedeschia aethiopica* (calla lily) Popović *et al*. 2017 | 1902 |
| *P. betavasculorum* | *Beta vulgaris* (sugar beet) Thomson *et al*. 1981<br>*Cynara scolymus* (artichoke) Gardan *et al*. 2003<br>*Helianthus* annuus (sunflower) Gardan *et al*. 2003<br>*Solanum tuberosum* (potato) Gardan *et al*. 2003 | 1981 |
| *P. brasiliense* | *Beta vulgaris* (sugar beet) Secor *et al*. 2016<br>*Brassica oleracea* var. acephala (leaf cabbage) Queiroz *et al*. 2017<br>*Capsicum annuum* (bell pepper) Gillis *et al*. 2017<br>*Cucumis sativus* (cucumber) Meng *et al*. 2017<br>*Cucurbita pepo* (zucchini) Moraes *et al*. 2017<br>*Cynara cardunculus* var. *scolymus* (artichoke) Italy Cariddi and Bubici 2016<br>*Nicotiana tabacum* (tobacco) Wang *et al*. 2017<br>*Solanum lycopersicum* (tomato) Rosskopf and Hong 2016<br>*Solanum tuberosum* (potato) Duarte *et al*. 2004<br>*Neobuxbaumia tetetzo* (cactus) Mexico Mejía-Sánchez *et al*. 2019 | 2004 |
| *P. cacticida* | *Carnegiea gigantea* (Cactus plant) Alcorn *et al*. 1991 (Hauben *et al*. 1999)<br>*Helianthus annuus* (sunflower) Valenzuela-Soto *et al*. 2015 | 1991 |
| *P. carotovorum* | *Abelmoschus gigantea* (okra) Nazerian *et al*. 2011a<br>*Artemisia absinthium* (wormwood) Tian *et al*. 2015<br>*Dieffenbachiae amoena* (dieffenbachia plant) Cetinkaya-Yildiz *et al*. 2007<br>*Brassica oleracea* (cabbage) Nazerian *et al*. 2011b.<br>*Cichorium intybus* (chicory) Lan *et al*. 2013<br>*Citrullus lanatus* (watermelon) Moloto and Goszczynska 2007<br>*Cucumis sativus* (cucumber) Nazerian *et al*. 2011c<br>*Cynara cardunculus* (cardoon) Gao *et al*. 2016<br>*Daucus carota* (carrot) Waleron *et al*. 2014 | 1901 |

| | | |
|---|---|---|
| | *Echonipsis chamaecereus (syn. Chamaecereus silvestrii)* (cactus) Kim *et al*. 2007<br>*Fritillaria imperialis* (crown imperial) Mahmoudi *et al*. 2007<br>*Haworthia* (haworthia) Baghaee-Ravari and Gerayeli 2015<br>*Ipomoea batatas* (sweet potato) Gao *et al*. 2016<br>*Kalanchoe tubilfora* (chandelier plant) Dahaghin and Shams-Bakhsh 2014<br>*Lactuca sativa* (lettuce) Nazerian *et al*. 2011b<br>*Lilium longflorum* (easter lily) Hahm *et al*. 2003<br>*Opuntia sp*. (cactus) Baghaee-Ravari and Gerayeli 2015<br>*Orostachys japonica* (rock pine) Cheon and Jeon 2014<br>*Orostachys malacophylla* (green duncecap) Kim and Jeon 2016<br>*Papaver somniferum* (opium poppy) Aranda *et al*. 2008<br>*Peperomia obtusifolia* (baby rubberplant) Dahaghin and Shams-Bakhsh 2014<br>*Peperomia ceparata* (emerald ripple peperomia) Dahaghin and Shams-Bakhsh 2014<br>*Plectranthus australis* (Swedish ivy) Dahaghin and Shams-Bakhsh 2014<br>*Pilea cadierei* (aluminium plant) Dahaghin and Shams-Bakhsh 2014<br>*Pinellia ternata* (crow-dipper) Ying *et al*. 2007<br>*Rheum x hybridum* (rhubarb) Waleron *et al*. 2014<br>*Silybum marianum* (milk thistle) Gao *et al*. 2014b<br>*Saintpaulia ionantha* (African violet) Dahaghin and Shams-Bakhsh 2014<br>*Solanum lycopersicum* (tomato) Caruso *et al*. 2016<br>*Solanum tuberosum* (potato) Hauben *et al*. 1998<br>*Spathiphyllum wallisii* (peace lily) Alippi and Lopez 2009<br>*Typhonium giganteum* (horn lian) Gao *et al*. 2014a | |
| *P. fontis* | waterways Oulghazi *et al*. 2019 | 2019 |
| *P. odoriferum* | *Allium ampeloprasum* (leek) Waleron *et al*. 2014<br>*Allium cepa* (onion) Waleron *et al*. 2014<br>*Apium graveolens* (celery) Waleron *et al*. 2014<br>*Brassica oleracea* (cauliflower) Cariddi and Bubici 2016<br>*Brassica rapa* (cabbage) Oskiera *et al*. 2017<br>*Cichorium endivia* (endive) Waleron *et al*. 2014<br>*Cichorium intybus* Gardan *et al*. 2003<br>*Daucus carota* (carrot) Waleron *et al*. 2014<br>*Ipomoea batatas* (sweet potato) Gao *et al*. 2016<br>*Petroselinum crispum* (parsley) Waleron *et al*. 2014<br>*Hyacinthus* sp. (hyacinth) Gardan *et al*. 2003 | 2003 |
| *P. parmentieri* | *Solanum tuberosum* (potato) Khayi *et al*. 2016 | 2016 |
| *P. peruviense* | *Solanum tuberosum* (potato) Waleron *et al*. 2018 | 2018 |
| *P. polaris* | *Solanum tuberosum* (potato) Dees *et al*. 2017 | 2017 |
| *P. polonicum* | groundwater Waleron *et al*. 2019 | 2019 |
| *P. punjabense* | *Solanum tuberosum* (potato) Sarfraz *et al*. 2018 | 2018 |
| *P. versatile* | *Solanum tuberosum* (potato) Portier *et al*. 2019<br>*Allium porrum* (leek) Portier *et al*. 2019<br>*Cichorium intybus* (chicory) Portier *et al*. 2019<br>*Synara scolymus* (artichoke) Portier *et al*. 2019<br>*Hyacinthus orientalis* (hyacinth) Portier *et al*. 2019<br>*Brassica oleracea* (gabbage) Portier *et al*. 2019<br>*Chrysanthemum* (chrysanthemum) Portier *et al*. 2019<br>*Cyclamen* (cyclamen) Portier *et al*. 2019<br>*Daucus carota* (carrot) Portier *et al*. 2019<br>*Iris* (iris) Portier *et al*. 2019<br>*Lactuca sativa* (lettuce) Portier *et al*. 2019<br>*Primula* (primula) Portier *et al*. 2019<br>waterways Portier *et al*. 2019 | 2019 |
| *P. wasabiae* | *Brassica oleracea* (gabbage) Golkhandan *et al*. 2013<br>*Eutrema japonicum* (Japanese horseradish) Goto and Matsumoto 1987, Gardan *et al*. 2003 | 1987 |

| | | |
|---|---|---|
| | *Ipomoea batatas* (sweet potato) Golkhandan *et al*. 2013<br>*Solanum lycopersicum* (tomato) Golkhandan *et al*. 2013<br>*Solanum melongena* (eggplant) Golkhandan *et al*. 2013 | |
| *P. zantedeschiae* | *Zantedeschia* (calla lily) Waleron *et al*. 2019 | 2019 |
| *Pectobacterium sp.* | *Erythrina indica* (coral tree) Sutra *et al*. 1999 | 1999 |

## 1.3. TAXONOMIC METHODS USED FOR DELINEATION OF *PECTOBACTERIUM* SPECIES

### 1.3.1. Biochemical characteristics

In the past, when the gene or genome level information was not available, microbiologist used morphological and phenotypic characters, such as colony morphology and growth requirements for bacteria classification, and also biochemical properties such as production of reducing sugars and assimilation of carbon sources were used for delineating bacteria species (Schaad *et al*. 2001). Also, the host plants from which the bacterial strains were isolated and the virulence properties of the bacterial strains were considered important for the identification of *Pectobacterium* species (Jones 1950). Based on the pathogenicity and host-plant origin, three species of soft rot bacteria were identified and included into *Erwinia* genus as species or subspecies, among them the bacteria we now know as *P. atrosepticum*, *P. carotovorum* and *P. betavasculorum* (Jones 1950, Thomson *et al*. 1981). The biochemical properties played a significant role in determining the *P. wasabiae* as a distinct species (Goto and Matsumoto 1987). Biochemical tests and the nutritional requirements of the bacteria are still used as one of the main methods for determining new bacterial species (Tindall *et al*. 2010). Today these analyses are often performed with BIOLOG plates which allows multiple phenotypic tests to be performed at the same time (Bochner 2009).

### 1.3.2. DNA-DNA hybridization

DNA-DNA hybridization (DDH) is a method which measures the genetic similarity of two bacterial strains by determining the hybridization degree of two DNA strands. Schildkraut *et al*. (1961) were the first ones to demonstrate that hybrid DNA forms if organisms are genetically related with their DNA base composition. Since then, for about 50 years, DDH has been the gold standard for delineating the bacterial species and classification of

prokaryotes. The requirement for strains to be considered as the same species based on DDH is that ≥70% of the DNA from the two strains reassociates with a ≤5°C difference in melting temperatures (Stackebrandt *et al*. 2002, Stackebrandt and Goebel 1994, Wayne *et al*. 1987). The disadvantages of the laboratory-based DDH are that results are not reproducible and they may vary depending on the reannealing temperatures or methods used in a test laboratory, public databases cannot be created based on DDH results, data exhange between laboratories is not possible, procedure is time-consuming and rapid prokaryotic identification is not possible (Chan *et al*. 2012, Gevers *et al*. 2005).

DDH experiments have been used for the characterization and delineation of several *Pectobacterium* species. It was used as one of the methods for the characterization of *P. odoriferum, P. actinidiae* and *P. aroidearum* (Gallois *et al*. 1992, Koh *et al*. 2012 and Nabhan *et al*. 2013). Today *in-silico* DDH (*is*DDH) methods have been replaced the traditional DDH in the genomic sequence comparison.

### 1.3.3. 16S rRNA and housekeeping genes

The invention of polymerase chain reaction (PCR) technique in 1980s (Mullis *et al*. 1986, Saiki *et al*. 1988) made the 16S rRNA gene the most popular genetic marker for taxonomic identification for a long time (Yarza *et al*. 2014). 16S rRNA gene sequence similarity values lower than 98.65% identifies strains as different species (Kim *et al*. 2014). In bacterial genome there might be several 16s rRNA genes and the number of 16S rRNA genes varies per species (Větrovský and Baldrian 2013). Větrovský and Baldrian (2013) stated that variable amount of 16S rRNA makes analyses based on 16S rRNA gene more complicated, because usually the 16S rRNA gene copies are not identical and their copy number increases the diversity of the sequences. It has been demonstrated that even if two bacterial species have a high level of 16S rRNA gene sequence similarity, DDH analysis may identify strains as different species with DDH value being lower than 70% (Ash *et al*. 1991, Rosselló-Mora and Amann 2001, Stackebrandt and Goebel, 1994). Thus, the 16S rRNA gene sequence homology as a taxonomic marker for species delineation is not usually beneficial method, but it is routinely used in taxonomic projects to identify bacterial genera and closest relatives due to simplicity of the analysis (Tindall *et al*. 2010).

When *Erwinia* genus was separated to three genera *Pectobacterium*, *Erwinia* and *Brenneria,* 16S rRNA sequence similarity and phylogenetic relatedness was used for the classification (Hauben *et al*. 1998). Later, 16S rRNA gene phylogeny was used for the elevation of *P. carotovorum*, *P. atrosepticum* and *P. wasabiae* to species level (Gardan *et al*. 2003). Phylogenetic trees based on 16S sequences have been used until recent years to describe new species, for example *P. actinidiae*, *P. polaris* and *P. aquaticum* (Koh *et al*. 2012, Dees *et al*. 2017, Pédron *et al*. 2019). Also, other phylogeny of housekeeping genes, such as gapA, has been used in verification of new *Pectobacterium* species, such as *P. punjabense* and *P. aquaticum* (Cigna *et al*. 2016).

### 1.3.4. Multilocus sequence analysis

Multilocus sequence analysis (MLSA) has been used to complement 16S rRNA gene-based analysis in order to obtain better phylogenetic definition of closely related bacterial species (Gevers *et al*. 2005). MLSA is a method where partial sequences of several protein coding genes are concatenated and used to calculate phylogenetic trees (Glaeser and Kämpfer 2015). Concatenated aligned sequences are more informative in bacterial phylogenetic than only a single gene information. Suitable genes for use in the MLSA phylogeny are orthologous genes, genes which are ubiquitous for the taxon under study and are present in a single copy (Gevers *et al*. 2005, Vinuesa *et al*. 2005, Vinuesa *et al*. 2008). Charlebois and Doolittle (2004) stated that choosing of several housekeeping genes for MLSA, such as *gyrB* (DNA gyrase, beta subunit), *rpoD* (RNA polymerase, $\sigma^{70}$ factor), *dnaK* (chaperone protein DnaK), *trpB* (tryptophan synthase, beta subunit), and *recA* (recombinase A), are suitable because they encode core metabolic enzymes. For the analysis, the DNA or protein sequences can be selected. One close relative strain known to be outside of the group of the interest is selected for the root of the tree (Huelsenbeck *et al*. 2002). In addition to the outgroup method, other methods such as molecular clock and nonreversible models of substitution may be used to root phylogenetic trees, and of these, the outgroup method has been shown to describe the root of the tree consistently (Huelsenbeck *et al*. 2002).

MLSA has been used in prokaryotic taxonomy to increase the accuracy of phylogenetic analyzes, but there is no specific procedure agreed how MLSA should be performed and how many and which genes should be chosen for the analysis. Nevertheless, the new species *P. aroidearum*, *P. parmentieri*, *P. polonicum*, *P. fontis* and *P. versatile* have been

described based on the MLSA analysis of 3-14 genes, and MLSA was also used for the elevation of *P. odoriferum*, *P. brasiliense* and *P. actinidiae* to the species level (Nabhan *et al*. 2013, Khayi *et al*. 2016, Waleron *et al*. 2018, Sarfraz *et al*. 2018, Waleron *et al*. 2019b, Oulghazi *et al*. 2019, Portier *et al*. 2019).

### 1.3.5. Genome-based methods

In recent years, sequencing the bacterial genomes has turned into a common practice. The first complete bacterial genome was sequenced in 1995 (Fleischmann *et al*. 1995), and after that sequencing technologies have improved and at the same time the costs have reduced. Various techniques are used to utilize information of the entire bacterial genome for taxonomy. Average nucleotide identity and digital DNA-DNA hybridization techniques are used to determine bacterial taxonomy (Auch *et al*. 2010b, Konstantinidis and Tiedje 2005). For genome level calculation, several programs exist, e. g. ANI tool by Kostas lab (Goris *et al*. 2007), Pyani (Pritchard *et al*. 2016), Genome-to-Genome Distance Calculator (GGDC) (Auch *et al*. 2010a) and JSpecies (Richter *et al*. 2015).

Method called *in-silico* DNA-DNA hybridization (*is*DDH) is based on the overall genetic similarity of the two genomes, where the local alignment is performed to identify high-scoring segment pairs, and these comparisons are converted to a single genome-to-genome distance value (Meier-Kolthoff *et al*. 2013). This was the first method that utilized the entire bacterial genome to determine prokaryotic taxonomy. The resulting distance values mimic the wet-laboratory hybridization results, providing an easy alternative to the traditional time-consuming DDH experiments. The analysis can be performed without specific knowledge with the user-friendly Genome-to-genome distance calculator (GGDC) program online (https://ggdc.dsmz.de/home.php). The results obtained can be compared with each other with a cut-off value of 70% for species delineation as in traditional DDH (Auch *et al*. 2010a). However, according to Auch *et al*. (2010a), the genome distance calculation based on the whole genomes is also dependent on the software used and the settings of the program.

Average nucleotide identity (ANI) is used to determine the genetic relatedness of prokaryotic species, and it is a second standard *in silico*-method that has replaced DDH analysis in bacterial taxonomy (Richter and Rosselló-Móra 2009). ANI is a method in which nucleotide identity of coding regions of two genomes is calculated (Jain *et al*. 2018) and ANI values

have strong correlation with DDH values (Colston *et al.* 2014, Goris *et al.* 2007). The range of 95-96% average nucleotide identity value corresponds to the 70% DNA-DNA hybridization (DDH) cut-off value (Wayne *et al*. 1987). Several studies have been published of ANI and DDH relationship. Goris *et al*. (2007) have shown close relationship between DDH and ANI values and strong correlation for DDH and ANI values in regression analysis and according to them, ANI cut-off value for species is 95 ± 0.5% and it corresponds 70% DDH for species delineation. Also, Chan *et al*. 2012, Konstantinidis and Tiedje 2005 and Wayne *et al*. 1987 have shown that ANI values of 95-96% equals to 70% DDH value. Figueras *et al*. (2014) have shown that ANI values obtained by different software tools differs. Of four ANI tools tested (JSpecies, Gegenees, http://www.gegenees.org, EzGenome, http://www.ezbiocloud.net/ezgenome/ani and the ANI calculator, http://enve-omics.ce.gatech.edu/ani/index), ANI calculator always provided higher values than the JSpecies and the EzGenome tools, but the range was dependent on the genomes tested (Figueras *et al*. 2014). The JSpecies and the EzGenome provided similar kind of results (Figueras *et al*. 2014). This means that even the algorithm used in computer programs is the same, the different programs may produce different results and methods used for phylogenetic analyses should be better treated as good approximations of the results (Leighton Pritchard, personal communication).

Since 2016, ANI analysis and digital DNA-DNA hybridization techniques have been routinely used for the characterization and delineation of new *Pectobacterium* species and verification of the taxonomic position of the previous ones as part of the polyphasic strategy for taxonomic description of bacterial species (Ramasamy *et al*. 2014). The taxonomic status of many *Pectobacterium* isolates originally identified by their biochemical properties and 16S rRNA gene sequences has changed when the genomic relationships have been re-evaluated using ANI and *is*DDH (Zhang *et al*. 2016). As a result, culture collections and sequence databases contain many misidentified and wrongly named isolates of *Pectobacterium*, which has further complicated the taxonomic studies and diagnostics of *Pectobacterium* species.

### 1.4. WHOLE GENOME SEQUENCING OF PACTOBACTERIA

The whole genome sequencing (WGS) of the prokaryotic bacteria is nowadays common because costs have decreased. The number of *Pectobacterium* genomes at public online

databases has been growing fast. In addition, new research methods based on genomic information have been developed for taxonomic research, and with them new bacterial species can be efficiently identified. The number of recognized *Pectobacterium* species and new genomes have increased rapidly. At the time of writing this thesis, there are about 150 *Pectobacterium* genomes available at the National Centre for Biotechnology Information (NCBI). They represent 18 recognized or proposed *Pectobacterium* species: *P. actinidiae, P. aquaticum*, *P. aroidearum* (appears under another name, *P. c*. subsp. *carotovorum* as originally described in NCBI), *P. atrosepticum*, *P*. *betavasculorum*, *P. brasiliense*, *P*. *cacticida* (has been removed from the online databases), *P*. *carotovorum, P*. *fontis*, *P. odoriferum P*. *parmentieri*, *P*. *peruviense*, *P*. *polaris*, *P*. *polonicum*, *P*. *punjabense*, *P*. *versatile*, *P*. *wasabiae* and *P*. *zantedeschiae* (Dees *et al*. 2017, Gardan *et al*. 2003, Goto and Matsumoto 1987, Hauben *et al*. 1998, Khayi *et al*. 2016, Nabhan *et al*. 2013, Oulghazi *et al*. 2019, Pédron *et al*. 2019, Portier, *et al*. 2019, Sarfraz *et al*. 2018, Waleron *et al*. 2018, Waleron *et al*. 2019a and Waleron *et al*. 2019b). However, *P. peruviense* and *P. zantedeschiae* are without valid acceptance according to the guidelines of the International Code of Nomenclature of Prokaryotes (ICNP, formerly the International Code of Nomenclature of Bacteria, ICNB), known as Prokaryotic Code (formerly Bacteriological Code) and they do not appear on the The List of Prokaryotic Names with Standing in Nomenclature (LPSN, https: http://www.bacterio.net, Parte 2014). The taxonomic identities of the species in the NCBI database is unreliable as many bacteria have been redefined and found to be of another species (Portier et al. 2019). This is especially true for *P. carotovorum* strains published before 2019, which may have comprised *P*. *actinidiae*, *P*. *aquaticum*, *P*. *brasiliense*, *P*. *odoriferum*, *P*. *polaris* and *P*. *versatile* bacterial species. Taxonomy of these species has recently been redefined by Portier et al. 2019. The same applies to *P*. *wasabiae* bacterial strains determined before 2016, as the bacterial strains may also belong to *P*. *parmentieri* species as stated by Khayi et al. 2016, especially if the strain has been isolated from potatoes.

## 1.5. APPROVAL OF NOVEL SPECIES OF BACTERIA

Approved List of Bacterial Names was published in the International Journal of Systematic Bacteriology (IJSB) in January 1980 (Skerman *et al*. 1980) and corrected version in 1989 (Skerman *et al*. 1989). Prokaryotic names that are valid should be included in the Approved lists pubslished in International Journal of Systematic and Evolutionary Microbiology

(IJSEM) (earlier IJSB) or in the Validation lists that are published on LPSN database available online at http://www.bacterio.net (Euzeby 1997, Parte 2014, Parte 2018). LPSN, which was originally called as The List of Bacterial Names with Standing in Nomenclature (LBSN), was set up by Jean Euzeby in 1997 who also maintained the database until he retired in 2013, and since then Aidan Parte has been responsible for the database (Euzeby 1997, Parte 2014). LPSN is funded by several private supporters (Parte 2018). The International Committee on Systematics of Prokaryotes (ICNP) regulates the naming of bacterial species (Lapage *et al*. 1992, Parte 2004, Parte 2018).

The name to be validly published should fulfill some basic criteria (Parte 2018). The first criterion requires the publication of the new bacterial species in the IJSEM journal or in the Validation list (Parte 2018). The Validation list is published in IJSEM, in which prokaryotic names not validly published are confirmed (Parte 2014). IJSEM accepts new taxons published in some other peer reviewed journals, if a request with the appropriate description of the strain is submitted to IJSEM (Tindall *et al*. 2006). According to second criterion, the taxon must be described in the publication of the species or it should be referred to the previous publication (Parte 2018). The third criterion requires a naming of the taxon (Parte 2018). The type strains of the new species published should also be placed into two public culture collections by the authors (Tindall *et al*. 2006, Parte 2018).

# 2. AIMS OF THE STUDY

The main focus of this research was to characterize *Pectobacterium* isolates found in Finland. Previously identified and characterized model strains were studied to verify their characteristics and taxonomic position and newly identified *Pectobacterium* isolates were characterized to verify their virulence and taxonomy. Biochemical features of the bacteria were determided to help in bacterial classification. Genome sequences were used to determine the taxonomical status of the species.

The study can be divided into three parts:

1. Determination of the biochemical characteristics of the bacterial strains
2. Characterization of their differences in the virulence, secretion systems, autoinducer molecules produced and ability to cause HR on susceptible plants
3. Utilization of gene and genome-level information for phylogenetic analyses and genome-level comparisons on bacteria to determine their taxonomic status

# 3. SUMMARY OF MATERIALS AND METHODS

The methods used in this study are listed in Table 3. Details of the methods are described in the original publications.

**Table 3. Summary of the methods used in this study.**

| Method | Publication |
|---|---|
| Average nucleotide identity analysis | IV |
| Bacterial cultivation | I-II-III-IV |
| Bacterial competition assay | IV |
| Bacterial DNA extraction | III-IV |
| Biochemical analysis | I-II-III-IV |
| Biolog plate assay | IV |
| Cellulase plate test | III |
| Citrate test | IV |
| Hypersensitive response assay (HR) | III-IV |
| Liquid chromatography-mass spectrometry (LC-MS) | III |
| Multilocus sequence analysis (MLSA) | II-III-IV |
| Polymerace chain reaction (PCR) | III |
| Potato tuber inoculation | III-IV |
| Sequence alignment | II-III-IV |
| Thin-layer chromatography (TLC) | III |
| Virulence assay | III-IV |

# 4. SUMMARY OF RESULTS AND DISCUSSION

## 4.1. CHARACTERIZATION OF THE STRAIN SCC3193

The soft rot pathogen *Pectobacterium* sp. SCC3193 was isolated from a diseased potato stem in early 1980s in Finland (Pirhonen *et al*. 1988). It has been a model organism used in the study of virulence of soft rot bacteria for over three decades. It was originally characterized to belong to *P. c.* subsp. *carotovorum*, but when the genome of SCC3193 was sequenced and characterized, its taxonomic status was reanalyzed. A phylogenetic tree based on housekeeping genes verified that it was closely related to *P. wasabiae* type strain CFBP 3304T. Biochemical characteristics of the strain SCC3193 were analyzed to study its similarity with the *P. wasabiae* and *P. atrosepticum* type strains to verify its taxonomic status (I). The analyzes were performed according to a standard biochemicals protocols defined in De Boer and Kelman (2001), Hyman *et al*. (2002) and Schaad *et al*. (2001). Several standard biochemical assays were performed, including growth in +37°C, phosphatase activity, indole production, erythromycin sensitivity, salt tolerance in 5% NaCl, production of reducing sugars from sucrose, assimilation of carbon sources (lactose, melibiose, raffinose and sorbitol) and utilization of α-methylglucoside (I and III) (Gallois *et al*. 1992, De Boer and Kelman 2001, Gardan *et al*. 2003). Strain SCC3193 differed from the *P. wasabiae* type strain CFBP 3304T in that the former assimilated melibiose and raffinose but the latter did not, but all other biochemical assays performed gave the same result for these strains. In addition, strain SCC3193 differed from the *P. carotovorum* type strain CFBP 2046T and *P. atrosepticum* type strain HAMBI 1429T due to its inability to grow at +37°C or in 5% NaCl, and production of reducing sugars from sucrose and fermentation of α-methylglucoside. Although there were differences in biochemical properties between SCC3193 and *P. wasabiae* type strain CFBP 3304, the strain SCC3193 was concluded to belong to *P. wasabiae* species (I). Later, when taxonomic position of potato-infecting *P. wasabiae* strains were re-evaluated, genome comparison separated a novel species *P. parmentieri* from the original Japanese horseradish *P. wasabiae* isolates (Khayi *et al*. 2016 and IV). The results show that the *P. parmentieri* has been present in Finland for a long time but was first incorrectly identified as *P. carotovorum* and then as *P. wasabiae*.

## 4.2. CHARACTERIZATION OF THE STRAIN SCC1

The bacterial strain SCC1 was isolated in Finland in 1980s from a diseased potato tuber and identified as belonging to *P. carotovorum* species (Saarilahti and Palva 1986). Since then, it has been used as a model organism to study the virulence of soft rot bacteria and plant-pathogen interactions. Strain SCC1 was initially identified as *P. carotovorum* because its biochemical properties, such as growth at +37 °C and in 5% NaCl, sensitivity to erythromycin, inability to produce indole, ability to assimilate lactose, melibiose, raffinose but not sorbitol and inability to produce reducing sugars from sucrose and acid from α-methyl glucoside, did not differ from the *P. carotovorum* type strain CFBP2046T (III).

To further characterize SCC1 strain, its whole genome was sequenced and seven housekeeping genes (*dnaN*, *fusA*, *gyrB*, *recA*, *rplB*, *rpoS* and *gyrA*) were used to determine the taxonomic status. The genes used in the analysis were selected based on their use in other MLSA assays used for *Pectobacterium* phylogeny (Nabhan *et al*. 2011, Moretti *et al*. 2016, Waleron *et al*. 2018). Type strains of *P. atrosepticum*, *P. betavasculorum*, *P. parmentieri*, *P. wasabiae*, *P. actinidiae*, *P. brasiliense*, *P. carotovorum*, *P. odoriferum* and *P. aroidearum* were included in the analysis and *D. solani* was used as a root of the tree. Phylogenetic analysis placed SCC1 strain as a separate branch in the same clade with the other *P. carotovorum* strains (*P. actinidiae*, *P. brasiliense*, *P. carotovorum*, *P. odoriferum*), with *P. odoriferum* being its closest relative (II). Nevertheless, the identity of the strain SCC1 could not be verified at the time of the analysis (II). Later Shirshikov *et al*. (2018) performed comparative genomic studies with *Pectobacterium* species and identified SCC1 as a new species and tentatively named it with other related strains as *Candidatus* Pectobacterium maceratum. Following taxonomic studies and thorough characterization of these strains, SCC1 and similar strains were recently renamed as *P. versatile* (Portier *et al.* 2019, IV). The result show that *P. versatile* has existed in Finland for a long time but was incorrectly identified as *P. carotovorum*.

## 4.3. CHARACTERIZATION OF *PECTOBACTERIUM* ISOLATES IDENTIFIED IN FINLAND 2004

Bacteria samples were collected from rotten potato stems and tubers in Finland in 2004 to identify the reason for increased problems caused by blackleg and soft rot of potato. Strains

were isolated from symptomatic samples obtained from 39 fields and 23 tuber lots, and semi-selective CVP medium was used for isolation of bacteria (Laurila *et al*. 2008). From some of the samples *D. solani* was identified and interpreted to be the reason for increased blackleg and soft rot problems in Finland, but also numerous *Pectobacterium* isolates were isolated and characterized further in the present study (III, IV). The 16S-23S intergenic spacer (IGS) sequence of the strains were sequenced and identified in BLAST analysis and strains were tentatively determined to belong to *Pectobacterium* genus (III). To study their taxonomic position, IGS sequences were generated and used for BLAST analyses and for grouping of the isolates in phylogenetic tree (III). Furthermore, a MLSA tree was made based on the concatenated sequences of 16S rRNA gene, IGS region, *acnA* and *mdh* genes for taxonomic identification of the *Pectobacterium* isolates by comparison to *P. atrosepticum, P. betavasculorum, P. brasiliense, P. carotovorum, P. odoriferum* and *P. wasabiae* type strains. Also, two Dutch isolates identified as blackleg-causing *P. carotovorum* (de Haan *et al*. 2008) were included in the MLSA analysis (III). Three groups were identified in the IGS and MLSA trees, one of them consisted mainly of strains isolated from potato tubers and clustered closest to *P. carotovorum*. One of the other two groups contained one Finnish isolate and the Dutch isolates, and clustered with*. P. parmentieri* (*P. wasabiae*) type strain in MLSA analysis, suggesting that *P. parmentieri* was present also in the Netherlands but was wrongly identified as *P. carotovorum* (III). Furthermore, some isolates clustered in the same branch with *P. carotovorum* but were separated from it with high bootstrap value, which made their taxonomic position unclear (III). These atypical strains, s0416, s0417, s0421, s0424 and s0425, had been isolated from rotten stem samples that originated from three locations in Finland. These isolates were studied further to characterize their virulence and taxonomic position.

The atypical strains s0416, s0417, s0421, s0424 and s0425 were subjected to conventional and real time PCR tests designated to detect *P. atrosepticum, P. brasiliense*, *P. carotovorum, P. wasabiae* and *Dickeya* strains from seed lots. In these tests, these five bacterial strains did not give positive result with any primer pairs tested, which gave indications they did not belong to any one of the tested species (IV). However, their phenotype, such as growth at +37 °C and in 5% NaCl, ability to assimilate lactose, melibiose, raffinose but not sorbitol and inability to produce reducing sugars from sucrose and acid from α-methyl glucoside, did not differ from the *P. carotovorum* type strain CFBP2046T, thus they were initially identified as *P. carotovorum* (III).

It had been suggested earlier that various *P. carotovorum* isolates differed from each other in the production of autoinducer molecules (de Haan *et al*. 2008). To identify differences between the Finnish isolates, their ability to produce autoinducers was studied with liquid chromatograph-mass spectrometry (LC-MS) assay, bioassay on cellulase plates and with thin-layer chromatography (TLC) experiments (III). Autoinducer characterization revealed that the *P. carotovorum* isolates differed based on the production of the autoinducer molecules. It was found that the isolates s0416, s0417, s0421, s0424 and s0425 did not produce detectable levels of autoinducers after 24 hours of growth and after 5 hours the amounts were poorly detectable, whereas the other isolates produced higher amounts of autoinducer molecules. However, concentration of the s0416, s0417, s0421, s0424 and s0425 samples showed that isolates in the both groups produced mainly 3-oxo-C6 homoserine lactones as autoinducers.

It had also been suggested that isolates identified as *P. carotovorum* differed in the presence and absence of T3SS genes (Kim *et al*. 2009). Harpin-encoding gene *hrpN*, present in the T3SS cluster of *P. atrosepticum,* was used as a probe in Southern blot analysis to determine the presence of this gene in *P. carotovorum* isolates. The results showed that s0416, s0417, s0421, s0424 and s0425 isolates lacked homologous sequences to the *hrpN* gene, whereas most of the typical *P. carotovorum* isolates showed homologous band (III). To study the presence of the whole T3SS in the isolates, PCR amplification with primers specific to *hrcC*, *hrcL* genes present in the T3SS cluster, and *hecB* gene, flanking the T3SS gene cluster, were used to confirm that s0416, s0417, s0421, s0424 and s0425 lacked typical T3SS gene cluster present in most *P. carotovorum* isolates (III). Furthermore, the ability of the isolates to cause HR in tobacco plants (*Nicotiana tabacum* cv. Xanthi and *Nicotiana benthamiana*) was tested. The isolates s0416, s0417, s0421, s0424 and s0425, and SCC3193 used as negative control, failed to cause HR in *N. tabacum* and *N. benthamiana* leaves. This result suggested that the isolates identified as atypical *P. carotovorum* lack the T3SS gene cluster, whereas the typical isolates harbor it (III, IV). The virulence of isolates representing both identified *P. carotovorum* groups was studied in field conditions with inoculated seed tubers. The result showed that none of the isolates selected from the two groups of bacteria identified as *P. carotovorum* could cause blackleg, which was surprising because the atypical strains had been isolated from diseased potato stems.

## 4.4. IDENTIFICATION OF *P. POLARIS* SUBSP. *PARVUM*

To further characterize the atypical *Pectobacterium* isolates identified in Finland, two strains, s0416 and s0421 were selected for genome sequencing. BLASTN analyses performed in NCBI with 16S rRNAgene sequences obtained from their genomes showed that their 16S sequences were identical with each other and with previously sequenced *P. polaris* isolates NCPPB 3395, isolated from potato in the Netherlands, and Y1, isolated from *Brassica rapa* in China (IV). Comparison of the 16S rRNA gene sequences present in NCPPB 3395, Y1, s0416 and s0421 to *Pectobacterium* type strains showed that their 16S rRNAgene sequence was 99.55% similar to the 16S rRNAgene sequence of the *P. polaris* type strain NIBIO 1006$^T$, suggesting that s0416 and s0421 belong to newly identified *P. polaris* species. 16S sequence of s0416 and s0421 was also 99.22-98.70% similar to 16S sequences of type strains of *P. versatile, P. carotovorum, P. brasiliense, P. aquaticum, P. odoriferum, P. actinidiae* and *P. wasabiae*, showing that 16S sequences between many *Pectobacterium* species are above the suggested 98.65% cut-off for species delineation (Kim *et al*. 2014) (IV).

Average Nucleotide Identity (ANI) values were determined with Pyani (Pritchard *et al*. 2016) for s0416, s0421, NCPPB 3395 and Y1 isolates and all presently known 18 *Pectobacterium* type strains. Finnish isolates s0416 and s0421 had less than 95% pairwise similarity value with *P. carotovorum*, *P. brasiliense, P. odoriferum* and all the other *Pectobacterium* species included in the analysis, except *P. polaris* strains (IV). As a cut-off value for species identification in ANI analysis, the value of 95%-96% has generally been considered (Richter and Rosselló-Móra 2009, Pritchard *et al*. 2016). Finnish isolates s0416 and s0421 showed 96% with *P. polaris* type strain NIBIO1006$^T$ and NIBIO 1392. *P. polaris* NIBIO 1006$^T$ and NIBIO 1392 shared 97% ANI value with each other, while Finnish strains s0416 and s0421 shared over 99% ANI values with *P. polaris* NCPPB 3395 and Y1, suggesting the *P. polaris* isolates can be divided into two groups. Seven housekeeping genes (*dnaN*, *fusA*, *gyrB*, *recA*, *rplB*, *rpoS* and *gyrA*) were used to determine the phylogenetic relationship of the Finnish isolates and four *P. polaris* strains available in the NCBI database, including the type strain of *P. polaris* NIBIO1006T. The genes were selected for the MLSA analysis based on their use in the other assays for *Pectobacterium* phylogeny (Nabhan *et al*. 2011, Moretti *et al*. 2016, Waleron *et al*. 2018). All *Pectobacterium* type strains available in the NCBI database were added to the analysis. The results showed that the *P. polaris* isolates

clustered into two clades, *P. polaris* isolates s0416, s0421, NCPPB 3395 and Y1 in one and the Norwegian isolates *P. polaris* NIBIO 1006$^{T}$ and NIBIO 1392 in the other, separated with high bootstrap value (Figure 1). Taken together, ANI and phylogenetic analysis results suggest that strains s0416, s0421, NCPPB 3395 and Y1 may represent a novel subspecies of *P. polaris*. Because the subspecies should have differences in the phenotype as well, the novel isolates and their genomes were characterized further.

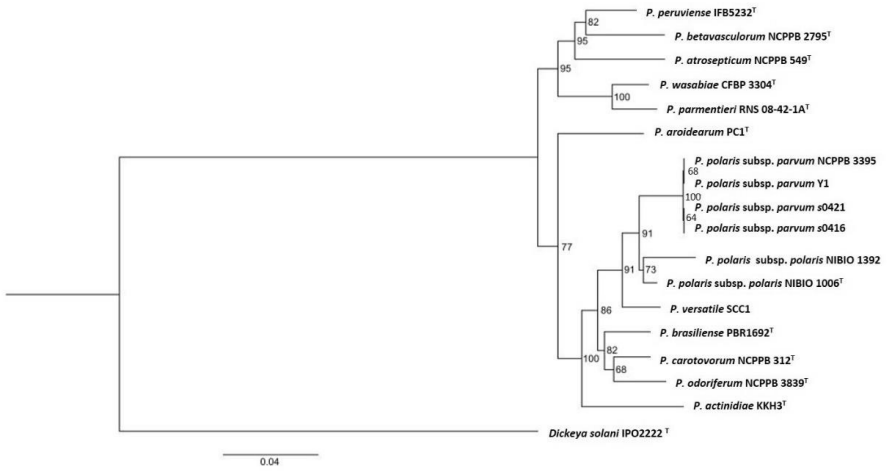


Figure 1. A phylogenetic tree showing the evolutionary relationship of Finnish isolates s0416 and s0421, named *P. polaris* subsp. *parvum* in the present work, to *P. p.* subsp. *parvum* NCPPB3395 and YCT1, and *Pectobacterium* type strains, including the type strain of *P. polaris* NIBIO1006T. The tree was built from seven concatenetaed housekeeping genes (*dnaN, fusA, gyrB, recA, rplB, rpoS* and *gyrA*). Bootstrap values from 1000 replicates are shown in each branch. *D. solani* IPO2222 was used as the outgroup. T after the strain name point out the type strain. The scale bar indicates 0.04 substitutions per nucleotide position.

Because *P. polaris* strains appeared to differ amongst each other, their genomes were compared. Strains s0416, s0421, NCPPB 3395, NIBIO 1006$^{T}$ and NIBIO 1392 were included in the genomic comparison but Y1 strain was excluded because its biological informativeness in the comparison could have been low due to the exceptionally short genome and because strain had not been included the RefSeq database by the NCBI. With the help of Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) secondary

metabolite clusters were determined (Medema *et al*. 2011). Analysis revealed systematic differences between analyzed strains. Similarity was found with the s0416, s0421 and NCPPB 3395 gene clusters in gene organization and sequence to the genes required for production of phenazine antibiotic D-alanylgriseoluteic acid (AGA) by *Enterobacter agglomerans* Eh1087 (Giddens *et al*. 2002). The BLASTN program confirmed the result, and showed that *P. polaris* NIBIO 1006$^T$, NIBIO 1392 or none of the other *Pectobacterium* strains present in NCBI database contained the gene cluster. Phenazines are small compounds often with broad-spectrum antibiotic activity pruduced by many bacteria (Turner and Messenger 1986). Furthermore, AntiSMASH analysis further revealed that bacterial strains s0416, s0421 and NCPPB 3395 contained also a gene cluster for production of ß-lactam antibiotic, whereas NIBIO 1006$^T$ and NIBIO 1392 lacked this cluster. According to Zeng and Lin (2013), penicillin binding protein (PBP) is an enzyme involved in bacterial cell wall biosynthesis and ß -lactam antibiotics are able to bind and acylate the active site of it, which in turn causes the bacterial cell lysis and death (Zeng and Lin 2013). ß-lactam genes present in s0416, s0421 and NCPPB 3395 resembled in sequence and in organization the beta-lactam antibiotic, carbapenem, which has been shown to be produced by *P. carotovorum* (McGowan *et al*. 2005).

The presence of various secretion systems in the bacterial genomes were determined with the Type Three, Four and Six secretion system Hunter (T346Hunter) program (Martínez-García *et al*. 2015) and manually with BLASTN program. All *P. polaris* genomes, except Y1, were compared with T346Hunter, and two T3SS gene clusters were detected in all of them, one of them related to production of flagella and the other one involved in injection of virulence proteins into the host cells. A closer examination of the non-flagellar T3SS genes with BLASTN revealed that the *P. polaris* NIBIO 1006$^T$ and NIBIO1392 strains contained the typical Hrp/Hrc T3SS gene cluster found in most *Pectobacterium* strains. Interestingly, s0416, s0421 and NCPPB 3395 had an atypical T3SS resembling SPI-1 present in several *Pantoea* and *Erwinia* species. SPI-1 T3SS is required by *P. stewartii* subsp. *stewartii* and *Salmonella enterica* to persist in their insect vectors (flea beetle and leafhopper, respectively) (Correa *et al*. 2012, Dundore-Arias *et al*. 2015). Isolates s0416, s0421 and NCPPB 3395 are the first examples in the *Pectobacterium* genus to contain SPI-1-like T3SS. Furthermore, s0416, s0421 and NCPPB 3395 genomes harbored a VirB-like T4SS involved in effector translocation, but no T6SS, whereas the NIBIO 1006$^T$ and NIBIO 1392 genomes lacked the VirB region but possessed a T6SS (IV).

The ability of the Finnish *P. polaris* strains to use different carbon substrates as an energy source was studied on Biolog Phenotype Microarray PM01 and PM02 plates at the Veterinary Laboratories Agency, UK. Results showed that *P. polaris* strains s0416, s0417 and s0421 were not able to use citrate as their energy source, which was confirmed with Simmons' citrate media test. Simmons' citrate media consist of both sodium citrate as the sole source of carbon and ammonium dihydrogen phosphate as the sole source of nitrogen (Simmons 1926). Five Finnish strains (s0416, s0417, s0421, s0424 and s0425), three *P. carotovorum* isolates (s0427, t0437 and t0438), *P. polaris* strains NIBIO 1006$^T$ and NCPPB 3395, *P. carotovorum* CFBP 2046$^T$, *P. odoriferum* CFBP 1878$^T$, *P. brasiliense* CFBP 6617$^T$, *P. atrosepticum* ICMP 1526$^T$, *P. wasabiae* CFBP 3304$^T$, *P. betavasculorum* CFBP 1539$^T$, *P. cacticida* CFBP 3628$^T$, *P. parmentieri* SCC3193 and *D. solani* s0432-1 were tested. Simmons' citrate test confirmed the results obtained in the Biolog analysis and showed that the Finnish *P. polaris* strains and NCPPB 3395 were all negative in the analysis, but *P. carotovorum* type strain, Finnish *P. carotovorum* isolates and *P. polaris* type strain all gave positive results in the citrate test (IV) (Figure 2).

The virulence of *P. carotovorum* and *P. polaris* type strains and three Finnish *P. polaris* isolates was tested on potato tubers. All the bacterial strains were capable of rotting potato tuber tissue, but there were differences between bacterial strains in their pathogenicity. The Finnish *P. polaris* isolates and NCPPB 3395 had statistically significantly lower virulence than *P. carotovorum* CFPB 2046$^T$ and *P. polaris* NIBIO 1006$^T$ type strains (Figure 2). Furthermore, the ability of *Pectobacterium* species to produce antibacterial metabolites against other *Pectobacteria* on M9 minimal induction media. Interestingly, *P. polaris* isolates s0416, s0417, s0421, s0424, s0425 and NCPPB 3395 showed antibacterial activity and were able to inhibit especially the growth of the *D. solani* strains but also other *Pectobacterium* species, including *P. carotovorum*, *P. brasiliense* and *P. cacticida* type strains (data not shown) (Figure 2). This toxic molecule was not produced by the *P. polaris* type strain NIBIO 1006$^T$ (IV).

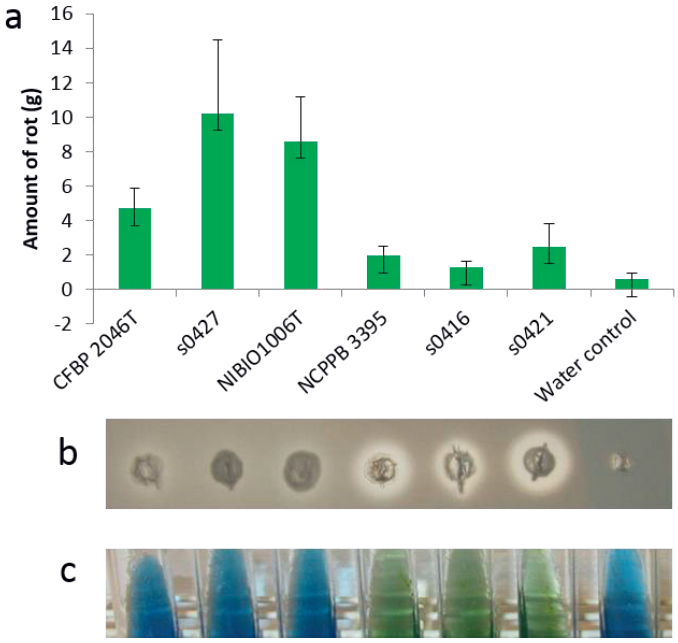


Figure 2. a) The amount of rotten potato tuber tissue caused by *P. carotovorum* type strain 2046T, Finnish *P. carotovorum* s0427, *P. polaris* type strain NIBIO1006T, NCPPB3395, the Finnish isolates identified in the present work as *P. polaris* subsp. *parvum*, and water control. b) Inhibition of the growth of *D. solani* on M9 minimal induction media. c) Citrate test with Simmons' citrate media, where the green color indicates citrate-negative result and the blue color indicates citrate-positive result. Bacterial strains are in the same order in all panels.

*P. polaris* strains isolated from the potato stems and NCPPB 3395 isolated from potato plants in The Netherlands, and possibly also Y1 isolated from *Brassica rapa* in China, seem to be closely related to *P. polaris*, but form a separate group in ANI, have differences in the genomic clusters and secretion systems and have a difference in the citrate utilization, virulence and production of toxic metabolites against closely related bacteria. Based on these differences, they can be described as a novel subspecies, here named as *P. polaris* subsp. *parvum*. A citrate-negative phenotype of the *P. polaris* subsp. *parvum* may explain why the they did not cause blackleg in the field assay and have low virulence in potato tubers. According to Nies and Brown (1998), iron has a central role in catalytic biochemical reactions, and Urbany and Neuhaus (2008) stated that citrate is needed for iron uptake in bacteria and the ability of the bacterium to infect the plant may be limited if there is no iron available. Citrate uptake is required for the full virulence of *P. atrosepticum* during growth in

plant tissue (Urbany and Neuhaus 2008). It is possible that *P. polaris* subsp. *parvum* isolates may be secondary pathogens which arrive to rotten potato stems with insects from other cultivated or wild plants and invade existing damaged tissue caused by other pathogens. In the plant, they may outcompete the other bacteria with production of toxic metabolites. To characterize the possibility of insect transmission, attempts to isolate similar strains from *D. melanogaster* were performed, but no bacteria were identified. Bacteria may be carried also with the other insect species as Rossmann *et al*. (2018) have shown. NCPPB 3395 (synonym strain 196) was isolated in the Netherlands from *Solanum tuberosum* by H. Maas Geesteranus, supposedly sometime during 70s (De Boer at al. 1979), which suggests that *P. polaris* subsp. *parvum* isolates have been present in Europe for a long time but misidentified as *P*. *carotovorum*.

# 5. CONCLUDING REMARKS

Soft rot bacteria isolated from rotten potato stems and tubers and originally identified as *P. carotovorum* were characterized in this study with phylogenetic and genetic tools. Among the characterized strains were SCC3193 and SCC1 identified early 80s, and since then used as model pathogens to study virulence of soft rot bacteria, as well as strains isolated from potato stems in 2004. The results show that several of the strains previously included into the *P. carotovorum* belong to other *Pectobacterium* species, among them SCC3193 that was identified as *P. wasabiae* (now *P. parmentieri*) and SCC1 that could not be identified but has later included into *P. versatile*. Furthermore, some of the strains isolated in 2004 were observed to resemble a newly identified *P. polaris*, from which they differed by phenotypic characteristics, and therefore, they were concluded in this work to form a new subspecies, *P. polaris* subsp. *parvum*. These strains were isolated from diseased potato stems in Finland but they could not cause blackleg, and contrary to *P. polaris* type strain, their virulence in potato tubers was low, suggesting that their main ecological niche may not be potato. Furthermore, they differed from *P. polaris* type strains also by their ability to produce toxic metabolites against other soft rot bacteria, and by T3SS that resembled the T3SS in *Salmonella* and *Pantoea* species that interact with insects. These results suggest that *P. polaris* subsp. *parvum* isolates may have a closer contact with insects than the other soft rot bacteria. The reason why many *P. carotovorum* strains were previously misidentified may be due to the fact that *Pectobacterium* species were initially often identified by few biochemical assays, 16S rRNA gene sequence similarities or phylogenetic analyses. In recent years, genome-wide comparative methods have been adopted as a taxonomic tool, providing a much more detailed picture of similarities and differences compared to previous methods. In the present taxonomy, it is almost necessary to use whole-genome level information for comparative studies to be sure about the taxonomic positions of the characterized *Pectobacterium* isolates. Due to the changes in the taxonomic positions of many strains, previously included especially into *P. carotovorum* and *P. wasabiea* species, sequence data repositories and culture collections contain numerous wrongly named sequences and strains, which may complicate taxonomic and diagnostic studies of soft rot Pectobacteria.

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